bio-protocol Preprint

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PKG-dependent TSC2 phosphorylation

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An abbreviated version of this protocol was published in Life Science Alliance in Mar 2022

Single serine on TSC2 exerts biased control over mTORC1 activation mediated by ERK1/2 but not Akt

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Detailed protocol

1. Isolate and culture neonatal rat cardiomyocytes (NRCMs) at 1 million cells per well in six-well plates for 24 hours in DMEM with 10% FBS and 1% penicillin/streptomycin at 37°C, 5% CO_2. **Full Protocol for this below.**
2. Remove media and replace with fresh DMEM with 10% FBS and 1% penicillin/streptomycin and incubate for an additional 48 hours at 37°C, 5% CO_2
3. Remove media and replace with fresh DMEM with 1% penicillin/streptomycin (no FBS) and incubate for 24 hours at 37°C, 5% CO_2
4. Add appropriate inhibitor (SCH772984, 10 µM; MK-2206, 150 nM; DT3, 1 µM) or vehicle DMSO and incubate for 1 hour at 37°C, 5% CO_2 without a media change
5. Add appropriate hormone (ET-1, 100 nM; insulin, 10 µg/mL) or vehicle water and incubate for 15 minutes at 37°C, 5% CO_2 without a media change
6. Remove media and wash cells twice with cold PBS
7. Add cold lysis buffer supplemented with protease and phosphatase inhibitor to each well and collect the NRCMs by first dislodging the cells from the surface with a cell scrapper and second with a pipette to place the collected cells and buffer into a clean microcentrifuge tube
8. Lyse cells (still in lysis buffer) on ice with a sonicator
9. Centrifuge the lysed samples to separate into the soluble and insoluble fractions
10. Collect the soluble fraction in a clean microcentrifuge tube
11. Determine protein concentrations with BCA assay
12. Prepare samples in Li-Cor protein sample loading buffer to 2 µg/µL protein concentration
13. Run samples on TGX 7.5% gels at 100 volts for 2.5 hours
14. Transfer onto nitrocellulose membranes using a high molecular weight transfer program (Transblot turbo, Bio-Rad)
15. Rinse membrane in TBS
16. Block membranes in blocking buffer for 1 hour at room temperature on shaker
17. Remove blocking buffer and add p-TSC2 S1365 (mouse sequence, antibody raised in rabbit) antibody diluted in antibody dilutant 1:500 and incubate on shaker at 4°C overnight
18. Wash in TBS-T 3 X 10 minutes at room temperature on shaker
19. Incubate in anti-rabbit secondary antibody diluted in antibody dilutant on shaker at room temperature for 1 hour
20. Wash at room temperature in TBS-T 3 x 10 minutes on shaker followed by TBS 1 x 10 min
21. Image on infrared imaging system (Odyssey; Li-Cor)

Neonatal rat cardiac myocytes Isolation Protocol and Descriptions

KH Buffer:
Working solution 10X solution
NaCl 140mM 8.18g 81.8g
KCl 4.8mM 0.358g 3.58g
MgSO4 1.2mM 0.144g 1.44g
NaHCO3 4mM 0.336g 3.36g
NaH2PO4 1.2mM 0.144g 1.44g
HEPES 10mM 2.383g 23.83g
Glucose 12.5mM 2.25g - 0.25M Glucose 9g/200mL(4C)
DDW 1000mL 1000 mL (Store at 4C)
Working solution 10X solution 100mL + DDW 850mL + 0.25M Glucose 50mL
Adjust pH 7.4 and Filter, Store at 4C

Autoclave
Forceps 2
Scissors 1
Small beaker with small stirrer (30mL) 1

Small beaker with small bar (50mL) 1
Beaker (100mL) 2
0.1% gelatin (TypeA)
B) Collagenase typeII (Worthington) 1g/100mL KHBuffer, and filter
Put to stock tubes, 1.5mL each, store at -20C
C) Trypsin 2.5% (Gibco, 15090-046)
Put to stock tubes, 0.6mL each, store at -20C
D) DMEM (Gibco 11965-092)
E) Penicillin/streptomysin (Gibco 15140-122)
F) ITSx (Gibco 51500-056)

Pups

• Order E17 pregnant rat (normally delivered on Tue and do experiment on next Monday)
• Use 1 to 2-day old pups

Procedure

1) Coat culture dish with 0.1% gelatin. After aspiration and dry, wash with sterile PBS.
2) Prepare KHB 35mLX1 (per 20rat) 37C warm, 10mLX2 on ice (each in 50mL tube)
3) Dissolve Collagenase and Trypsin (each 1 stock tube) to pre-warmed 35mL KHB (Solution A)
4) Put 15mL KHB into 100mL-autclaved beaker and on ice.
5) 70% ethanol in 100mL-autoclaved beaker.
6) Prepare ether or isoflurene to anesthetize.
7) Anesthetize lightly 6-7 neonatal rats.
8) Take a rat with sterile forceps and moisten a rat in 70% ethanol
9) Attach the rat abdomen with another sterile forceps and cut the left border of the sternum with sterile scissors. Scissors should be placed from a left infrasternal angle and advanced along the chest wall. Bleeding is a sign of heart injury.
10) Move the forceps to lower edge of chest.
11) Catch the upper side of the chest with another forceps and push out the heart.
12) Cut the heart and drop into the ice cold KHB.
13) Throw the rat to trash bag.
14) Repeat 7)-13)
15)Wash the hearts twice with ice-cold KHB
16) Cut once each hearts with scissors. It may be easy to cut it on a sterile culture dish.
17) Move the cut hearts to small beaker.
18) Remove buffer and add fresh 2mL KHB.
19) Add 4mL solution A
20) Stir for 10min in 37C.
21) Take away the buffer.
22) Add 6mL solution A.
23) Stir for 15min in 37C
24) Take the buffer (digested myocardium solution) and put into a new 50mL tube. Mix with 6mL DMEM containing 10%FBS to stop the effect of collagenase/tripsin.
25) Add 6mL solution A and stir again.
26) Repeat 23)-25) until heart peaces become very small and white. (Usually around 4 times repeats.
27) Take away residual unsolved tissue from digested myocardium solution by pipetting.
28) Centrifuge at 800rpm for 5min.
29) Remove supernatant and resuspend the pellet with DMEM (10%FBS). Put the cell solution into culture bottle. (20 rat/10mL DMEM/1 culture bottle)
30) Culture in 5% CO_2 incubator for 90min.
31) Pat the bottle and take the supernatant. Sticking cell is mainly fibroblasts. Add the bottle new DMEM with 10% FBS without BrdU. This bottle is used for cardiac fibroblasts culture.
32) The supernatant put into a new culture bottle or 50mL tube. Add DMEM containing 10% FBS with BrdU (0.1mM). Count the cell number. Usually cells from 20 rat can be diluted 50mL DMEM.
33) After cell count, dilute with DMEM/10%FBS/BrdU to appropriate concentration. Usually plating with 5X105/mL cell should be 80-90% confluence.
34) Culture in 5% CO_2 incubator.
35) Culture medium can be exchanged after 24 hours if necessary.

Solutions/Cautions:

1. Don’t put more than 5-6 pups in the beaker containing Isoflurane.
2. Don’t put all of pups in 70 % EtOH at once and leave long time (do 2-3 pups quickly).
3. After removing the heart, wash and cut them (2-5 times depend on heart size or minced, normally 2-3 cut is enough) quickly.
4. After adding the solution, adjust the speed depends on numbers of heart.
5. Don’t add the solution to the cells directly but put on side of beaker and swirl the solution using pipette. (Don’t touch the heart using pipette).
6. Always keep the collagenase solution at 37oC.
7. Wash the cut heart and remove debris and blood clearly.
8. Don’t remove the mucus-like material after 2nd and 3rd digestion.
9. After pre-plating (1:30 to 2 hours), transfer the media containing cells and wash 2-3 times very carefully.

Table of Antibodies:

Antibody	Concentration	Company	Catalogue Number
Phospho-mouse TSC2 (S1365) antibody	1:500	NovoPro Labs	120718
Anti-rabbit secondary antibody	1:10,000	Li-Cor	926-32211

Table of Reagents:

Reagent	Company	Catalogue Number
DMEM	Gibco	11965118
FBS	Gibco	10438034
Pen/Strep	Thermo	15140122
SCH772984	Selleck Chemicals	S7101
MK-2206	Selleck Chemicals	S1078
DT3	Millipore	370655
DMSO	Sigma	472301
ET1	Sigma	E7764
Insulin	Sigma	I9278
Water	Quality Biological	351-029-721
Lysis Buffer	Cell Signaling Technology	9803S
Phosstop	Sigma	4906845001
PMSF	Sigma	P7626
BCA assay kit	Pierce	100389
Loading buffer	Li-Cor	928-40004
7.5% TGX gels	Bio-Rad	456-1026
RTA transfer kit	Bio-Rad	1704271
TBS	Quality Biological	351-086-151
Tween	Sigma	P7949
Blocking buffer	Li-Cor	927-60003
Antibody dilutant	Li-Cor	927-65001

Table of Equipment:

Equipment	Company	Catalogue Number
Mini-protean tetra	Bio-Rad	1658004
Criterion	Bio-Rad	1656020
PowerPac	Bio-Rad	1645052
Transblot turbo	Bio-Rad	1704150

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Kass, D. (2022). PKG-dependent TSC2 phosphorylation. Bio-protocol Preprint. bio-protocol.org/prep1847.
2. Dunkerly-Eyring, B. L., Pan, S., Pinilla-Vera, M., McKoy, D., Mishra, S., Martinez, M. I. G., Oeing, C. U., Ranek, M. J. and Kass, D. A.(2022). Single serine on TSC2 exerts biased control over mTORC1 activation mediated by ERK1/2 but not Akt. Life Science Alliance 5(6). DOI: 10.26508/lsa.202101169